Enkephalin degrading enzymes are present in the electric organ of *Torpedo Californica*

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Two proteolytic activities that degrade [Leu^5]enkephalin were found in *Torpedo californica* electric organ. One is a soluble aminopeptidase that degrades enkephalin at the Tyr^1–Gly^2 peptide bond, and the second is an endopeptidase that degrades enkephalin at the Gly^3–Phe^4 peptide bond. The aminopeptidase is inhibited by low concentrations of puromycin and bestatin. More than 60% of the endopeptidase is associated with the particulate fraction and is almost completely inhibited by low concentrations of captopril (SQ 14225) or SQ 20881 (potent inhibitors of angiotensin converting enzyme). Thiorphan and phoshoramidon (potent enkephalinase inhibitors) are much less effective. The pattern of cleavage and inhibition of the particulate endopeptidase thus resembles that of angiotensin converting enzyme.

Enkephalin degradation *Electric organ membrane* *Aminopeptidase*
Angiotensin converting enzyme *Protease inhibitor* *Cholinergic synapse*

1. INTRODUCTION

Proteases in the nervous system are implicated in the regulation of membrane receptors [1], in modulation of synaptic transmission at the neuromuscular junction [2] and in the metabolism of peptidergic signals [3,4]. This last group is of special interest since many peptides are currently implicated in modulation of neuronal activity. One such modulatory neuropeptide is enkephalin, a naturally occurring opioid pentapeptide, which has a variety of biological functions in the central and peripheral nervous system (reviews [5–7]). Enkephalins affect neuronal transmission at the neuromuscular junction [8], and it has been shown that opiates modulate synaptic efficacy in the electric organ of *Torpedo* (D.M. Michaelson et al. in preparation) a tissue of embryonic muscle origin [9].

A number of proteolytic activities from the brain have been implicated in the degradation of enkephalins. These include enkephalin degrading aminopeptidase, a neutral metalloendopeptidase termed 'enkephalinase' and angiotensin converting enzyme [3,10–12]. However, no information is available on proteases which are capable of degrading enkephalin at the neuromuscular junction or in the fish electric organ. We report here the presence and properties of two enkephalin-degrading proteolytic activities which are present in the electric organ of *Torpedo californica*.

2. MATERIALS AND METHODS

[Leu^5]Enkephalin was supplied by Miles Yeda, Rehovot. Acetylcholine chloride, bestatin, puromycin dihydrochloride, phosphoramidon and catalase from bovine liver (twice crystallized) were purchased from Sigma St. Louis. Captopril (SQ 14225) and the nonapeptide SQ 20881 were kindly provided by Squibb Institute for Medical Research, NJ. Thiorphan was kindly donated by Dr B.P. Roques, University Rene Descartes, Paris. Decamethonium was obtained from ICN-K and K, Plainview, NY. α-[^{125}I]Bungarotoxin (αBT) (20 Ci/mmol) was prepared as in [13]. [*tyrosyl*-3,5-^{3}H]Enkephalin (5-L-leucine) (20 Ci/mmol) was obtained from Amersham Radiochemical Centre and [*acetyl*-^{3}H]acetylcholine iodide (90 mCi/mmol) from New England

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Nuclear. *T. californica* electric organ was obtained in frozen form from Pacific Bio-marine, Venice, CA.

2.1. *Preparation of crude membranes*

Frozen electric organ (15 g) was homogenized (Sorvall Omni-Mixer 4 × 0.5 min, maximal speed) in 3 vols of 10 mM Tris–HCl containing 1 M NaCl (pH 7.4). The suspension was filtered through cheese-cloth and centrifuged at 1000 × *g* for 10 min. The crude homogenate was centrifuged at 30000 × *g* for 60 min, the supernatant (S_{30}) collected and the crude membrane pellet (P_{30}) dissolved in 1.5 vols of 2 mM Tris–HCl (pH 7.4). In several experiments the crude homogenate was sedimented for 60 min at 100000 × *g*, the pellet dissolved in 10 mM Tris–HCl (pH 7.4) (Tris buffer) and resedimented under the same conditions (P_{100} membranes). Protein was determined as in [14].

2.2. *Sucrose gradient centrifugation*

For sucrose gradient centrifugation the P_{100} membranes obtained from 25 g tissue were suspended in 5 ml Tris buffer containing 5% sucrose (w/w) and layered over 5 ml of 20% sucrose (w/w) on top of a continuous 26 ml 29–40% (w/w) sucrose gradient. All sucrose solutions contained 10 mM Tris (pH 7.4). The gradient was centrifuged for 7 h at 80000 × *g* in an SW 27 rotor at 5°C and 1 ml fractions were collected.

2.3. *Enzymatic assay*

Enzymatic hydrolysis of [Leu^5]enkephalin was assayed essentially as in [15]. Briefly, mixtures of labeled and unlabeled [Leu^5]enkephalin (70000 cpm, 1 μM final concentration) were incubated with the enzyme for 30 min at 30°C in a final volume of 100 μl containing 10 mM Tris–HCl (pH 7.4). Incubation was stopped by the addition of acetic acid (1 M final) and the hydrolysis products were separated from undegraded enkephalin either by filtration of the reaction mixtures through Porapak Q columns, to which enkephalin but not tyrosine, Tyr-Gly or Tyr-Gly-Gly were adsorbed [16], or by thin-layer chromatography (silica plates in ethyl acetate–isopropanol–acetic acid–water, 40:40:1:19). Under the conditions tested, the hydrolysis of enkephalin did not exceed 30%.

Acetylcholinesterase activity was assayed as in [17]. Reaction mixtures (final volume 100 μl) contained 3 mM acetylcholine (84000 cpm), 120 mM NaCl and 50 mM Tris–HCl (pH 7.4). The reaction was carried out for 10 min at room temperature.

Binding of ^{125}I-αBT was performed as follows: ^{125}I-labeled αBT (8.5 nM, 30000 cpm) was incubated for 60 min at 30°C in a final volume of 100 μl containing 120 mM NaCl, 100 μg BSA and 50 mM Tris–HCl (pH 7.4). The reaction was terminated by filtration through EGWP cellulose acetate Millipore filters, as in [18]. Non-specific binding was determined in the presence of 0.1 mM decamethonium, and substracted from total binding.

3. RESULTS AND DISCUSSION

Homogenates of *T. californica* electric organ contain enzymes which degrade enkephalin. The thin-layer chromatography pattern of degradation of enkephalin by various subcellular fractions of the electric organ is shown in fig. 1. In the crude homogenate (panel A), the main proteolytic activity is that of an aminopeptidase which cleaves the enkephalin at the Tyr^1–Gly^2 peptide bond yielding free tyrosine. The second proteolytic activity is that of an endopeptidase cleaving the enkephalin at the Gly^3–Phe^4 peptide bond yielding Tyr-Gly-Gly. The aminopeptidase is a soluble enzyme and is responsible for 80% of the hydrolytic activity found in the S_{30} fraction (panel B). Only a residual aminopeptidase activity is associated with the crude membrane pellet (P_{30}, panel C). Further purification of the membranes, e.g. by means of sucrose gradient centrifugation, completely removed the aminopeptidase. The endopeptidase activity, on the other hand, was found in both the supernatant (30–40%) (panel B) as well as in the crude membrane fraction (60–70%) (panel C).

To characterize further the particulate endopeptidase activity, we have purified the crude membranes by a sucrose density gradient. As seen in fig. 2, most of the proteolytic activity was associated with membranes and separated into 4–5 peaks (panel A). A small part of the activity was soluble and migrated with a sedimention value lower than that of catalase (11.4 S). No major differences in the specificity of cleavage of the enkephalin molecules could be detected between the various peaks of the particulate and soluble en-

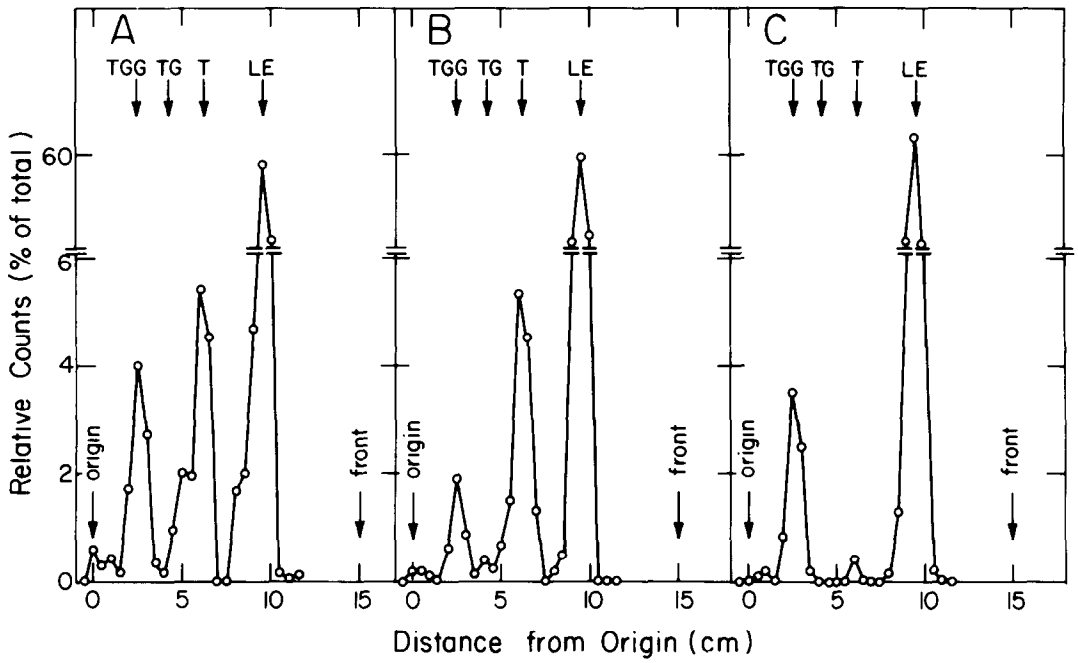

Fig. 1. Thin-layer chromatography of [Leu^5]enkephalin degradation products following a 30 min incubation with: (A) 5.6 μg crude homogenate (B). 3.6 μg S_{30}, (C) 5.7 μg crude membranes (P_{30}). Protein samples were dialyzed against 10 mM Tris buffer before assay. Markers were visualized by fluorescamine as in [16].

zyme (not shown).

The sucrose density gradient described above separates heavy membrane fractions containing acetylcholine receptor from lighter membrane fractions containing acetylcholinesterase (panel B). Our results demonstrate that the endopeptidase does not comigrate exclusively with either of these membrane fractions, but rather migrates at various sucrose densities indicating that the endopeptidase activity is not directly associated with any specific membrane fraction.

We have further characterized the two proteolytic activities by the use of selective inhibitors. The effect of a number of such inhibitors on the aminopeptidase is shown in table 1. Puromycin and bestatin are known to be very potent and selective inhibitors of rat brain enkephalin degrading aminopeptidase [19,20]. Puromycin (100 μM) and bestatin (10 μM) inhibited almost completely the activity of the electric organ aminopeptidase (90 and 86% inhibition respectively) and had no activity (bestatin) or low inhibitory activity (puromycin) toward the soluble endopeptidase. Thiorphan (10 μM), a potent inhibitor of the brain endopeptidase enkephalinase [21], did not affect the aminopeptidase activity but completely inhibited the soluble endopeptidase (94% inhibition). Similar results were obtained with the membrane associated endopeptidase. This enzyme (fig.3) was completely inhibited by 10 μM thiorphan (panel B) but not affected by 10 μM bestatin (panel C). Based on the identical cleavage pattern and the inhibitory study, it seems that the soluble and particulate endopeptidases are similar enzymes.

Two endopeptidases, i.e., enkephalinase and angiotensin converting enzyme (ACE), have been implicated in the degradation of the Gly^3-Phe^4 peptide bond of enkephalin [3,22]. Both enzymes are metalloenzymes but differ in their sensitivity to inhibition by various chelating compounds [23–26]. To distinguish between these two activities in purified membranes of electric organ, we used the following selective inhibitors: thiorphan and phosphoramidon, potent inhibitors of the brain enkephalinase but weaker inhibitors of ACE [25,26], as well as captopril and SQ 20881, potent inhibitors of ACE and poor inhibitors of enkephalinase [3, 27–29]. The results (table 2)

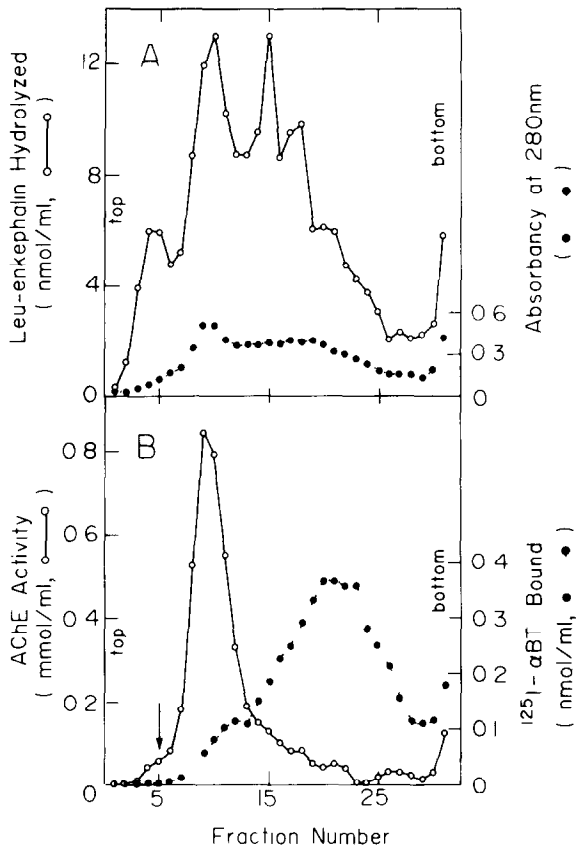

Fig. 2. Sucrose density gradient of *Torpedo* electric organ membranes. AChE activity, αBT specific binding and [Leu^5]enkephalin hydrolysis were determined using 0.1, 1 and 2.5 μl, respectively, from every fraction. The arrow indicates the position of catalase. AChE, acetylcholinesterase.

Table 1

The effect of various inhibitors on the hydrolysis of [Leu^5]enkephalin by proteolytic activities present in S_{30}

Inhibitor added	Inhibition* (%)	
	Amino-peptidase	Endo-peptidase
Puromycin (100 μM)	90	40
Bestatin (10 μM)	86	1
Thiorphan (10 μM)	0	94

*The enzymatic activity was determined by quantitative thin-layer chromatography of the reaction products

show that relatively low concentrations (30 nM) of captopril or SQ 20881 inhibited the enzyme very effectively (85 and 52% inhibition, respectively). Thiorpan and phosphoramidon at the same concentration were poor inhibitors (21 and >1% inhibition, respectively). At higher concentration (10 μM) phosphoramidon was still uneffective whereas thiorphan almost completely inhibited the enzyme (82% inhibition). This latter result agrees with the finding that thiorphan is more effective towards ACE than phosphoramidon [25,26]. Bestatin, which does not affect either of the endopeptidases mentioned above, did not inhibit the activity even at 10 μM. These results thus indicate that the endopeptidase present in *Torpedo* electric organ membrane resembles ACE.

In summary, we have found two proteolytic activities in *Torpedo* electric organ that are capable of degrading [Leu^5]enkephalin, a soluble aminopeptidase and a particulate endopeptidase. Based on the degradation pattern and inhibition studies, it seems that the aminopeptidase resembles the soluble aminopeptidase found in rat brain homogenates [18,19]. The membrane-associated enzyme seems to resemble ACE and differs from the rat brain membrane-associated endopeptidase enkephalinase.

The significance of such an enzyme in what is considered to be a pure cholinergic synapse is still not known. Since enkephalins have been implicated in the modulation of neuromuscular

Table 2

The effect of various inhibitors on the hydrolysis of [Leu^5]enkephalin by the endopeptidase present in washed membranes*

Inhibitor added	% Inhibition at	
	30 nM	10 μM
Captopril	85	85
SQ 20881	52	84
Thiorphan	22	82
Phosphoramidon	0	29
Bestatin	3	6

* Crude membranes (P_{30}) were resedimented at 30000 × *g* for 60 min and further purified on a flotation sucrose gradient as in [30] [10–54% sucrose (w/v) 12 h, 80000 × *g*, SW 27]. 3.4 μg protein of the purified membranes were used per assay

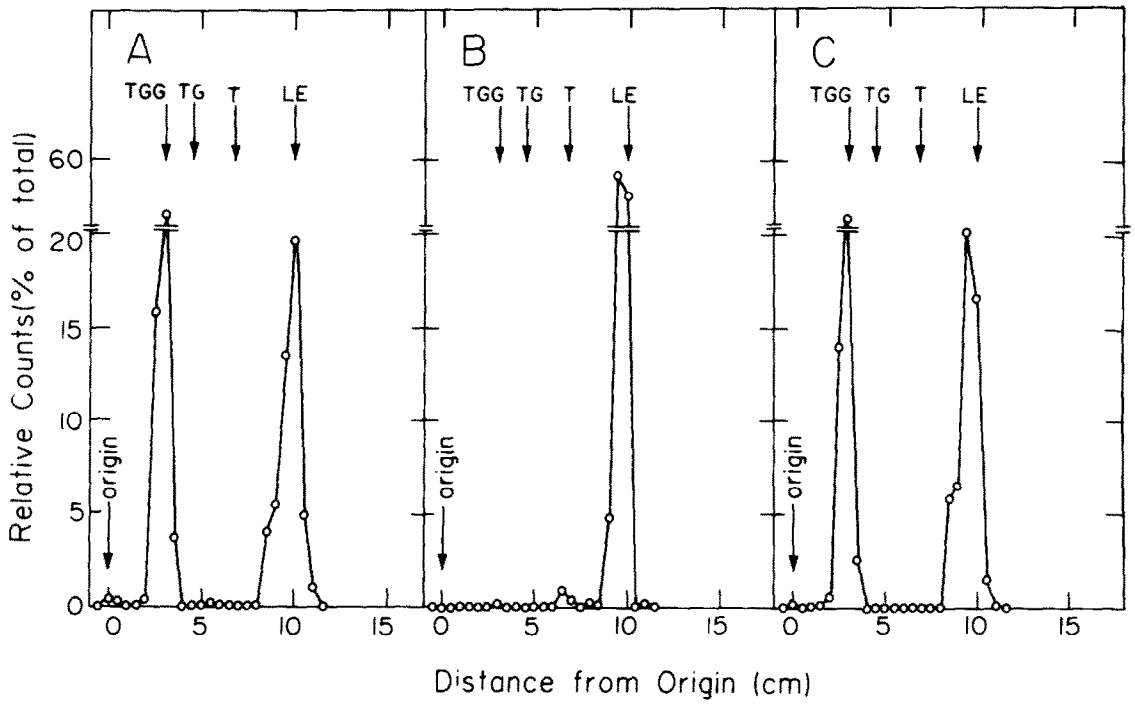

Fig. 3. Thin-layer chromatography of [Leu^5]enkephalin hydrolysed by 15.6 μg crude membranes (P_{100}) in the absence of inhibitors (A), in the presence of 10 μM thiorphan (B) and 10 μM bestatin (C).

transmission, enzymes which degrade enkephalins could play an important role in the synaptic transmission in muscle as well as in electric organ. On the other hand, there is also a possibility that ACE participates in the cleavage of other neuropeptides (e.g. angiotensin I, bradykinin) and it is of interest to find out whether such modulatory neuropeptides play a role in the function of the electric organ.

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